INFLUENCE OF PREFIXATION IN THE STUDY OF THE STRUCTURE OF SYMBIONTS OF *LOBARIA* SPP.

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SUMMARY. – In plants in general, the type of buffer used in fixation is apparently more important for changes in ultrastructural preservation than the type of fixing solution. In lichen thalli, it has been observed that glutaraldehyde used with phosphate buffer produces artefacts in the *Myrmecia* algal cells. The aim, therefore, was to determine whether these changes occur with different buffer concentrations, and to find another fixation method that does not produce these artefacts. In the present study, different concentrations of phosphate buffer are tried while the occurring ultrastructural changes are observed. The most important are cellular plasmolysis, presence of dense bodies that sometimes have a myelinic shape in the cytoplasm, and presence of dense half-moon structures near the plasmalemma. These changes occur from the lowest to the highest buffer concentration. Faced with the necessity to discard the buffer as a fixing vehicle in certain lichen thalli with *Myrmecia* phycobiont, the use of glutaraldehyde in bidistilled water is tested in two different conditions. This fixing procedure is fairly optimal when the algal symbiont is studied, whereas fixation in glutaraldehyde-buffer is more appropriate for the fungal component.

RÉSUMÉ. – Dans les plantes en général, pour la préservation des changements ultrastructuraux, le type de tampon utilisé pour la fixation est apparemment plus important que le type de solution de fixation. Pour les thalles lichéniques, il a été observé que la glutaraldéhyde utilisée avec du tampon phosphate, produit des artéfacts dans les cellules algales de *Myrmecia*. Il faut donc déterminer si ces changements apparaissent avec des concentrations différentes de tampon et trouver une autre méthode de fixation ne produisant pas d'artéfacts. Dans cette étude, différentes concentrations de tampon phosphate ont été essayées et nous avons observé les changements structuraux en résultant. Les changements les plus importants sont des plasmolyses cellulaires , la présence de corps denses présentant parfois une forme myélinique dans le cytoplasme, et la présence de structures denses en demi-lune près du plasmalemme. Leur apparition est corrélée avec l'augmentation de la concentration du tampon. Face à la nécessité d'éliminer le tampon pour la fixation de certains thalles lichéniques ayant le *Myrmecia* comme phycobionte, nous avons testé la glutaraldéhyde dans l'eau bidistillée sous deux conditions différentes. Cette méthode de fixation est optimale lorsque le symbionte algal est étudié, tandis que la fixation par la glutaraldéhyde dans le tampon est plus appropriée pour le composant fongique.

KEY WORDS : ultrastructure, glutaraldehyde, lichenized fungi, *Lobaria*, *Myrmecia*, mycobiont, phycobiont.

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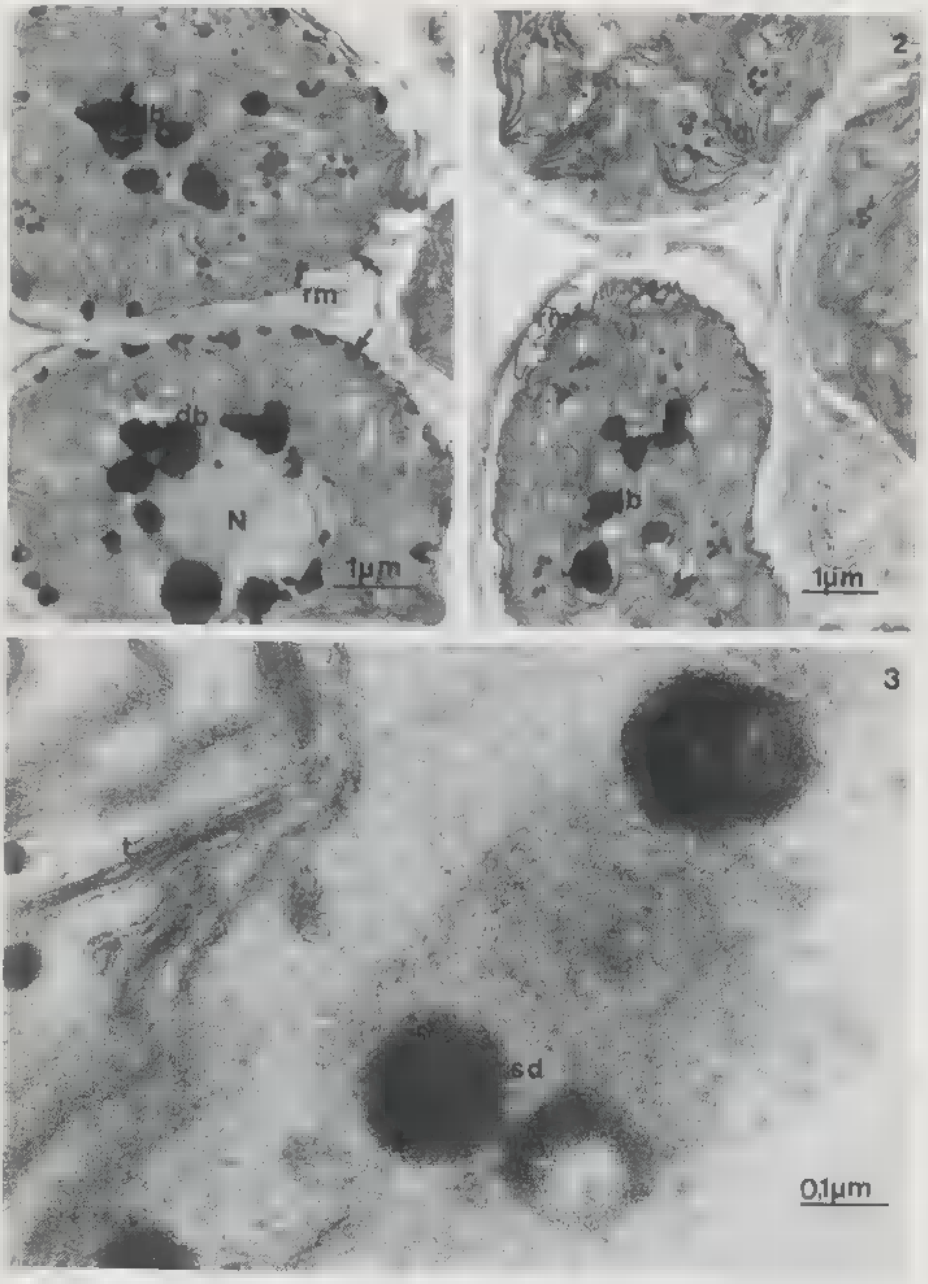

Plate 1 – Phycobiont (*Myrmecia*) in *Lobaria amplissima*. 1 : Samples fixed in glutaraldehyde in Sorensen buffer 0.2 pH 7.2. Note : half-moon structures beneath plasmalemma (arrows). 2 : Samples fixed in glutaraldehyde in Sorensen buffer 0.1 M pH 7.2. 3 : Samples fixed in glutaraldehyde in Sorensen buffer 0.05M pH 7.2. Note the presence of the storage droplets at the bottom.

Symbols – db : dark body, N : nucleus, rm : remainder membrane, sd : storage droplet.

INTRODUCTION

Few researchers have directed their attention to the development of appropriate methods to process plant material for transmission electron microscopy (COETZEE & VAN DER MERWE, 1984). During fixation, the differential permeability of the cell membranes decreases (IQBAL & WEAKLEY, 1974) and the presence of the pressure potential in plant cells may cause the loss of cell substance through this fixed membrane if enough pressure gradients exist. With plants in general, it seems that the type of buffer used for fixation is apparently more important in ultrastructural preservation than the type of fixing solution used (GOODCHILD & CRAIG, 1982).

In the preparation of lichen thalli for TEM, HOLOPAINEN (1982) observed that the effect of phosphate buffer concentration on ultrastructure varies with the time of the year. ASCASO & al. (1985) found that glutaraldehyde at 3.25% in phosphate buffer 0.05M produced changes in the ultrastructure of *Myrmecia* in *Lobaria amplissima*, which did not appear when the thallus was fixed in glutaraldehyde of the same concentration in water. These changes include varying size and density of the plasmalemma particles.

Before definitively discarding phosphate buffer as a vehicle for the glutaraldehyde fixing solution, the aim of the present study is to determine the influence of the buffer at different concentrations on the *Myrmecia* phycobiont in two lichen thalli. After observing (ASCASO & al., 1985) that the fixation in glutaraldehyde-water seems to be the most adequate, two different types of fixation were tried to decide upon the most appropriate.

MATERIAL AND METHOD

Thalli of *Lobaria amplissima* (Scop.) Forss. and *L. pulmonaria* (L.) Hoffm. were collected in April in Montejo de la Sierra near Madrid (Spain). The lichen samples were transported to the laboratory in polythene bags and immediately prefixed. The % water content by weight was 40 % and 35 % respectively, immediately prior to fixation.

Small sections of thalli, from 2-3 mm behind the lobe tips, were cut into small pieces and placed in 3.25 % glutaraldehyde in either 0.05 M, 0.1 M, 0.2 M phosphate buffer SORENSEN pH 7.2 or in distilled water. The fixation in glutaraldehyde with distilled water was made at the resulting pH 3.9 or after increasing the pH of the solution to 7.2.

Planche I – Phycobionte (*Myrmecia*) du *Lobaria amplissima*. 1 : Échantillons fixés par la glutaraldéhyde dans le tampon Sorensen 0,2M pH 7,2. Noter les structures en demi-lunes au-dessus du plasmalemme (flèches). 2 : Échantillons fixés par la glutaraldéhyde dans le tampon Sorensen 0,1M pH 7,2. 3 : Échantillons fixés par la glutaraldéhyde dans le tampon Sorensen 0,05M pH 7,2. Noter la présence de gouttelettes de réserve en bas. Symboles – db : corps noir, N: noyau, rm: membrane résiduelle, sd: gouttelette de réserve.

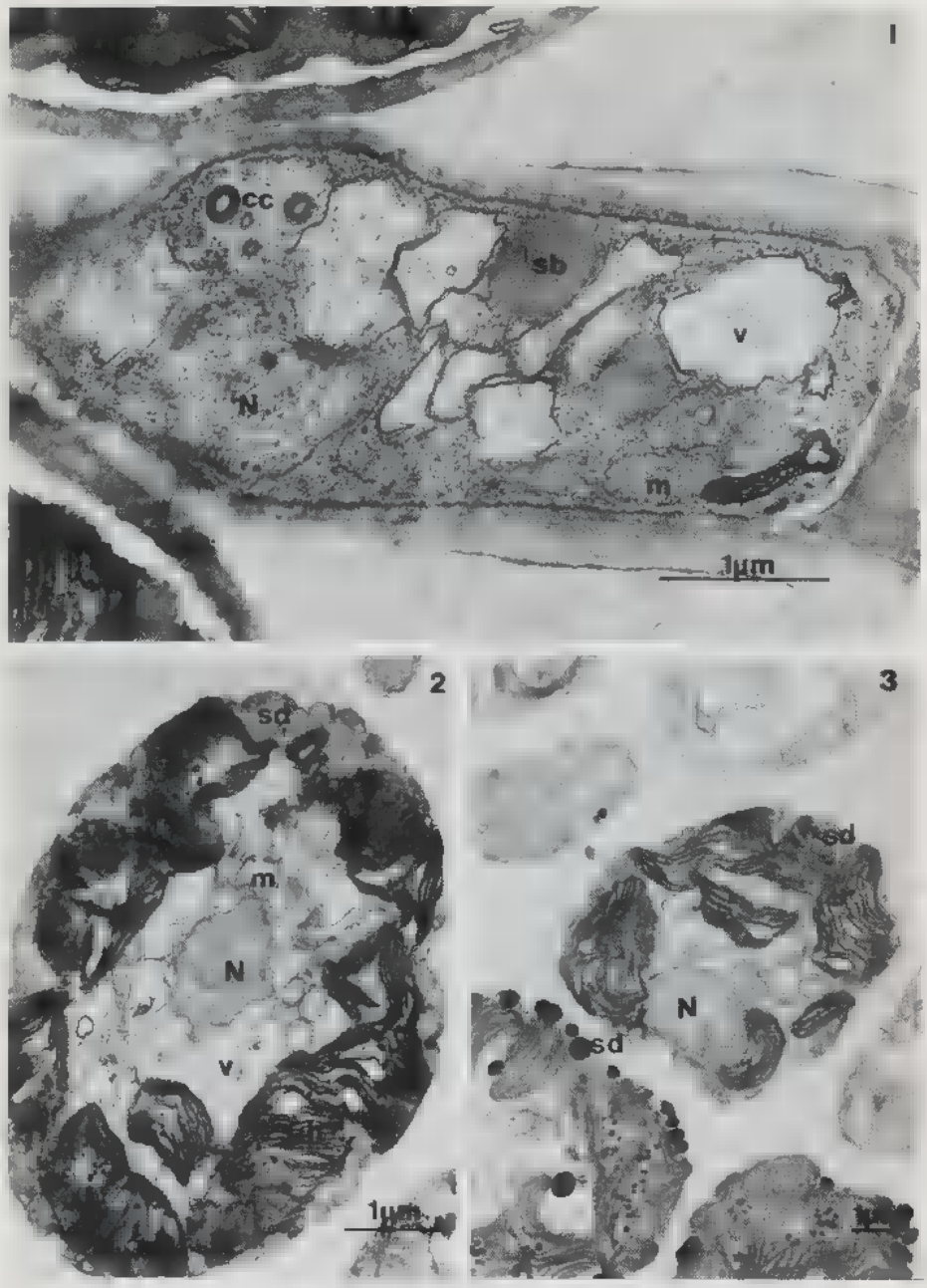

Plate II — 1 : Mycobiont in *Lobaria amplissima*. Samples fixed in glutaraldehyde in Sorensen buffer 0.1M. 2 : Phycobiont (*Myrmecia*) in *Lobaria amplissima*. Samples fixed in glutaraldehyde in distilled water. 3 : The same sample as in fig. 2, but fixed in glutaraldehyde-water at pH 7.2. Symbols — cc : concentric body, Ch : chloroplast, m : mitochondria, N : noyau, sb : storage body, sd : storage droplet, v : vacuole.

This prefixation was carried out at 4°C for 3 hours. The samples were then washed overnight in the corresponding buffer or water and postfixed in 1 % osmium tetroxide, dehydrated in 30 %, 50 %, 70 % and 100 % ethanol, embedded in SPURR resin (1969), sectioned and stained with REYNOLDS lead citrate (1963). Ultrathin sections were examined with the Philips EM 300 electron microscope. For image analysis, a MOP-Videoplan (Kontron) semiautomatic image analyser was used.

RESULTS AND DISCUSSION

The samples fixed in glutaraldehyde with 0.2 M buffer, show remarkable changes in the cytoplasm of the algal symbiont *Myrmecia* (pl. I, 1), both in the *Lobaria amplissima* and the *L. pulmonaria* thalli. In the cytoplasm area between the chloroplastic membrane and the nucleus, many dense bodies appear, some of which, when observed closely, look like myelinic shapes. Next to the plasmalemma, there are electron-dense structures that take the form of half-moons. A high degree of cell plasmolysis is also observed, and in some areas, there are membraneous remains in the empty space between the plasmalemma and the wall, which is a consequence of the plasmolysis. There also seems to be a certain degree of weakening (or perhaps disorganization) of the thylakoid laminas inside the chloroplast.

When glutaraldehyde diluted in 0.1 M buffer is used, the results obtained for the phycobiont (pl. I, 2) are similar, though of greater intensity since the different algal cells are affected in different ways.

The ultrastructural preservation is not adequate even when the buffer is used at a concentration of 0.05 M. In this case, there are fewer half-moon structures near the plasmalemma, while sizable storage droplets with high electronic density (pl. I, 3) are found. In the cytoplast spaces near the chloroplast still appear structures similar to myelinic shapes.

These changes seem to be due to movements of cell lipids in the phycobiont caused by the prefixation with buffer as a vehicle for glutaraldehyde. The lipids are stirred up during prefixation and are later fixed in the course of post-fixation with osmium tetroxide. As they are placed in certain areas of the cytoplasm, they give rise to the type of artefacts observed.

This is as far as the cell lipids are concerned. The effect that may exist on sugars, aminoacids and proteins is not known. According to COETZEE & VAN

Planche II — I : Mycobionte du *Lobaria amplissima*. Échantillons fixés par la glutaraldéhyde dans le tampon Sorensen 0,1 M. 2 : Phycobionte (*Myrmecia*) du *Lobaria amplissima*. Échantillons fixés par la glutaraldéhyde dans l'eau distillée. 3 : Même échantillon que la fig. 2, mais fixé par la glutaraldéhyde dans l'eau, à pH 7,2. Symboles - cc : corps concentrique, Ch : chloroplaste, m : mitochondrie, N : noyau, sb : corps de réserve, sd : gouttelette de réserve, v : vacuole.

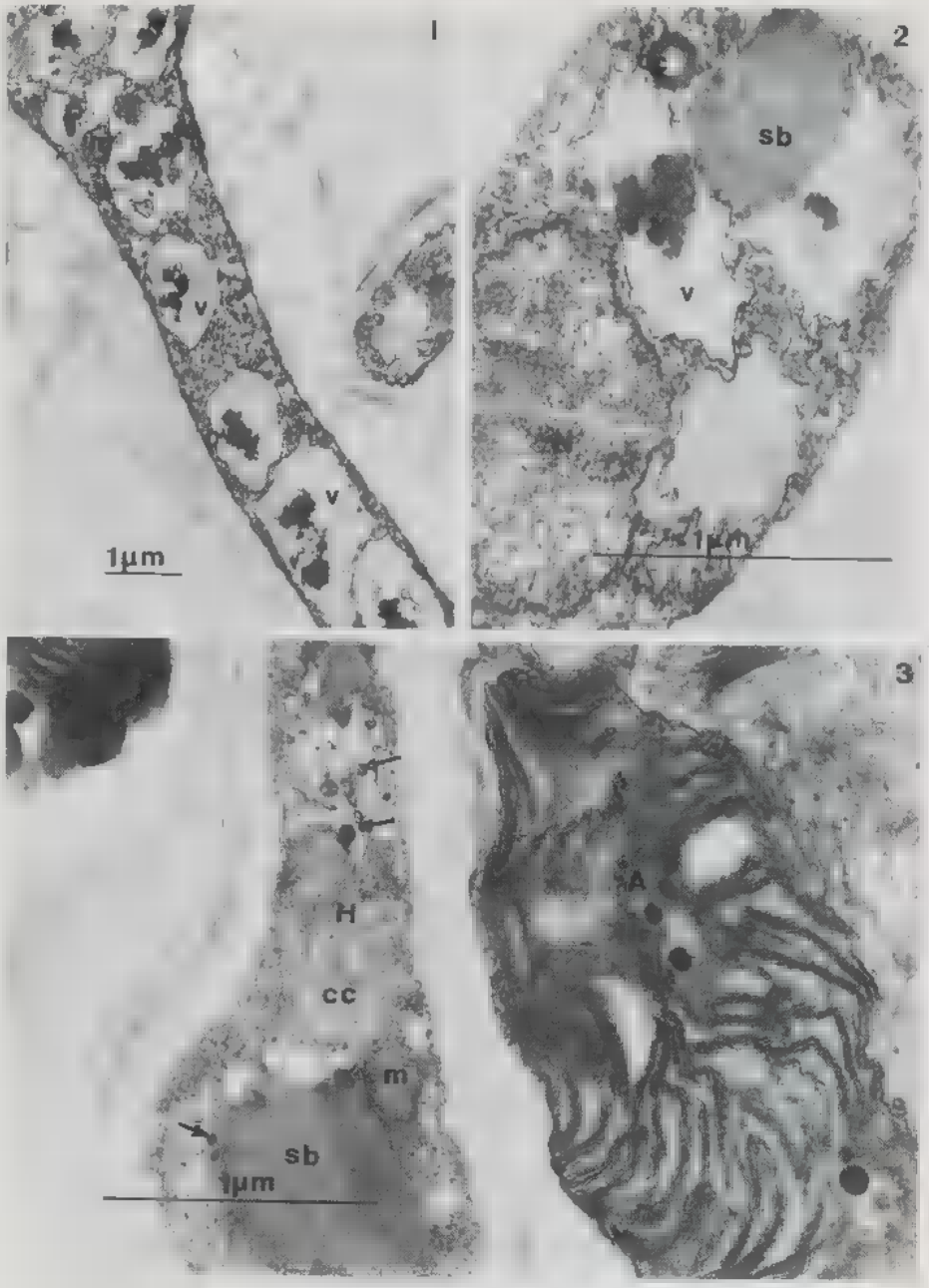

Plate III – 1 : Mycobiont in *Lobaria amplissima* fixed in glutaraldehyde in distilled water. 2 : Detail of the sample. 3 : Mycobiont in *Lobaria pulmonaria*. Note the dots or little globules in the cytoplasm (arrows). Fixed ■■ in fig. 1. Symbols - A : Algae, cc : concentric body, H : hyphae, m : mitochondria, N : nucleus, sb : storage body, v : vacuole.

DER MERWE (1984), the Na-Na phosphate buffer, even if it produces the lowest extraction per cent, induces a certain degree of extraction in these substances.

The half moon structures appear more frequently with increase of the buffer concentration; they seem to be very similar in electronic density and form to the dark polyphosphate bodies observed by HOLOPAINEN (1981) in *Trebouxia* of *Alectoria capillaris*. It is probable that the similarity between both structures is due not only to morphological but to chemical composition as well since if the dark polyphosphate bodies have a high phosphorus content, it may also be high in the half-moon structures in *Myrmecia* fixed with phosphate buffer.

In opposition to what was observed in algal cells, the general resolution of the organelles in the fungal cells is good in the ultrastructural study carried out with glutaraldehyde in phosphate buffer (pl. II, 1). No changes in the nucleus, mitochondria, vacuoles or cytoplasmic storage bodies seem to exist. The concentric bodies also have a normal appearance.

In samples prefixed in glutaraldehyde in bidistilled water, the phycobiont appears as shown in plate II, 2. No movement of lipids during prefixation is perceptible that may produce myelinic structures, nor are there half-moon structures near the plasmalemma. The thylakoid laminas look normal, as well as the reserve bodies found near the plasmalemma. In the area near the nucleus, there are vacuoles and mitochondria. These have an empty space inside which is surrounded by mitochondrial crests.

These results are similar to the ones obtained for the same species of *Myrmecia* phycobiont in other studies, in which a similar prefixation procedure was tested (ASCASO & al., 1985; ASCASO & al., 1986). As it can be observed in all microphotographs, there is no important cell plasmolysis.

When the same fixation is carried out on the samples, but raising the pH of the glutaraldehyde-water mixture to 7.2, the ultrastructural preservation is the one shown in plate II, 3. Through simple observation by TEM, this preservation (pl. II, 3) does not appear to be as good as the previous one (pl. II, 2), among other reasons, because of the electron-dense appearance of the storage droplets near the plasmalemma. Image analysis techniques were used to quantify these results (Tab. I).

Raising the pH to 7.2 in the glutaraldehyde-water mixture causes greater plasmolysis (2.8 μm^2 of space between plasmalemma and wall) as was found in the glutaraldehyde - distilled water mixture. In the first case, the size of the protoplast also increases. There are some dense bodies in the cytoplasm (2.77 %) as well as half-moon structures under the plasmalemma (5.65 %).

Planche III — 1 : Mycobionte du *Lobaria amplissima* fixé par la glutaraldéhyde dans l'eau distillée. 2 : Détail du même échantillon. 3 : Mycobionte du *Lobaria pulmonaria*. Noter les taches ou les petits globules dans le cytoplasme (flèches). Même fixation que pour la fig. 1. Symboles — A : Algue, cc : corps concentrique, H : hyphe, m : mitochondrie, N : noyau, sb : corps de réserve, v : vacuole.

	Glut+H_2O pH. 3.9	Glut+H_2O pH7.2	P <
Average area of protoplast (μm^2).	21.8±6.4	26.4±8.6	0.0
Area between plasmalemma and wall (μm^2)	1.1±0.4	2.8±1.4	0.0
Storage body area as per cent of protoplast area	5.07	4.38	
Dark body (1) area as per cent of protoplast area		2.77	
Half-moon shaped bodies under plasmalemma		5.65	

Table I — Measures of the different parameters in *Myrmecia* of *Lobaria amplissima*, prefixed in glutaraldehyde in water at resulting pH, or at pH raised to 7.2. (1) Myelinic shapes.

Tableau I - Mesures des différents paramètres pour le *Myrmecia* du *Lobaria amplissima*, préfixé par la glutaraldéhyde dans l'eau, au pH résultant, ou à pH 7,2. (1) Formes myéliniques.

This indicates that prefixation in glutaraldehyde-water is worse when the pH of the mixture is raised to 7.2. There seems to be a relationship between the presence of storage droplets and of half-moon structures. When the fixation is not very good, as in the case of glutaraldehyde-water pH 7.2, or is bad, as in the case of glutaraldehyde-buffer, the amount of structures counted as storage droplets becomes lower, while half-moon structures appear under the plasmalemma. This seems to indicate that the reserve bodies collapse, are transformed and become denser when fixation is bad.

Thus, the most appropriate fixation for the *Myrmecia* phycobiont in these thalli is glutaraldehyde-distilled water.

The effect of this type of fixation on the lichen mycobiont has been studied. In the mycobiont of *L. amplissima*, a vesiculation is observed all along the hypha (pl. III, 1). This vesiculation is not perceptible in the hyphae fixed in glutaraldehyde in phosphate buffer (pl. II, 1). Pl. III, 2, shows the ultrastructural changes that occur apart from high vacuolization. In the vacuoles, dense bodies are seen whose origin and structure are unknown. There is a possibility that in the mycobiont these may also correspond to lipid deposits. They do not have myelinic shapes like in the phycobiont, possibly due to the fact that in the phycobiont they may largely come from the fine thylakoid laminas, while this cannot be the case in the mycobiont which does not have such membranes.

Apart from the vacuoles and the dense deposits inside, a change in the cytoplasmic cytogel is observed, as well as in the nucleus, and even in the reserve bodies that are typical for these lichenized hyphae.

In the mycobiont of *L. pulmonaria* (pl. III, 3), there are also ultrastructural changes due to the effect of this fixation. They are of a very different nature and basically consist of the presence of dense points which sometimes reach the size of small globules in the cytoplasm.

From these observations on the mycobiont of *L. amplissima* and *L. pulmonaria* it can be concluded that although the glutaraldehyde fixation in water may be adequate for the *Myrmecia* phycobiont, it is not appropriate for the mycobiont of both thalli. The mycobionts of the two species react differently to the fixing solution.

ACKNOWLEDGEMENTS. – We wish to thank Teresa CARNOTA & Fernando PINTO for their technical assistance. The study was financed by the «Comisión Asesora de Investigación Científica y Técnica de la Presidencia del Gobierno» (the Government Advisory Commission for Scientific and Technical Research) as Project nº 169/85.

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